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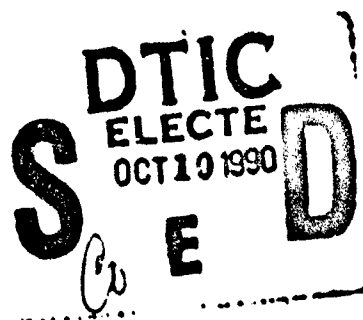
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MODE OF ACTION OF MEMBRANE PERTURBING AGENTS:
SNAKE VENOM CARDIOTOXINS AND PHOSPHOLIPASES A

FINAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) CTXs have at least two mechanisms of action. The first has a rapid onset and is reversible. The second has a prolonged latency to onset and results in cell death (cytolytic action). Free fatty acids in the membrane markedly affect CTX action. Melittin and CTX act by similar, but not identical mechanisms, based on similarities and differences in their interactions with PLA ₂ and differences in effects on lipid metabolism in cell cultures. Bee venom and snake venom PLA ₂ s differ markedly in their interactions with CTXs and melittin. The most consistent interaction (marked mutual synergism) between the membrane perturbing toxins (CTX and melittin) and any PLA ₂ is allowing the PLA ₂ enzyme to penetrate to formerly unavailable substrates. The <i>Naja naja kaouthia</i> CTX and melittin do possess activities not dependent on venom PLA ₂ contamination in the toxin fraction. These toxins both elevate free fatty acids and diglycerides by a mechanism unrelated to activation of endogenous PLA ₂ activity. These elevated neutral lipids appear related to activation of <i>de novo</i> synthesis of fatty acids, or breakdown of triglycerides, by the CTXs.					
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Problem Under Study:

Contract No. DAMD17-87-C-7155 addresses the mode of action of snake venom cardiotoxins (CTXs) and the interaction of these toxins with a second snake venom component, phospholipase A₂ (PLA₂). Also examined were bee venom melittin, an analogue of the snake venom CTX, and its interaction with bee venom PLA₂. Studies on presynaptically-acting snake venom PLA₂ neurotoxins examine whether similarities exist in mechanisms between these toxins and the CTXs, or if the CTXs act synergistically with the PLA₂ neurotoxins. CTXs are potent membrane perturbing agents and PLA₂s hydrolyze diacylphosphoglycerides at the two position, generating two biologically active metabolites - free fatty acids and lysophospholipids. The CTXs and PLA₂s act in synergy to induce hemolysis of red blood cells and skeletal muscle contractures. The CTXs and PLA₂s are of special interest due to the mutual potentiation observed; that is, the hemolytic activity of CTX is greatly increased by PLA₂ and the hydrolytic activity of PLA₂ is greatly increased by CTX. Combinations of these agents with other toxins, such as the presynaptically-acting snake venom neurotoxins, which all possess PLA₂ activity, would result in novel potent biological warfare approaches. Additionally, based on our recent studies, there are similarities between the modes of action of CTX and presynaptically-acting snake venom toxins. Therefore, a greater understanding of the modes of action of venom CTXs and PLA₂s and their interactions has important military significance.

The specific problems addressed in this contract are:

1. What are the effects of CTX alone and CTX-PLA₂ combinations on contracture induction of human and rat skeletal muscle and on Ca²⁺ transients in human and equine lymphocytes?
2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?
3. What are the hemolytic effects of CTX alone and CTX-PLA₂ combinations in red blood cells from different species?
4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?
5. What is the role of toxin internalization in the action of CTX?
6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA₂s?

Background:

Early studies in the isolation of protein components from snake venoms identified a number of toxic low molecular weight polypeptides (ca. 6000 MW). These toxins were identified in a number of laboratories and, because they affected a large number of systems, some confusion arose as to the true site of action of the toxins. The polypeptides were named according to their observed toxic actions and included: cardiotoxins, which arrested the heart in systole (Sarkar, 1951; Lee et al., 1968); Cobramine A and B (Larsen and Wolff, 1968); cytotoxins (Braganca et al., 1967) and; direct-lytic factors (Condrea et al., 1964). It later became apparent that these seemingly different toxins indeed shared similar mechanisms when compared on the same assay systems. The multitude of different effects possessed by each of these

toxins were suggested to be best described by the more general term membrane-active polypeptides (Condrea, 1974). However, the toxins have since been grouped together under the original name, cardiotoxin (CTX). CTXs are now regarded as low molecular weight basic polypeptides that, among other effects, depolarize and induce contractures in muscle (Chang, 1979) and hemolyze red blood cells (Condrea, 1974; 1979). There appears to be considerable amino acid sequence homology among the CTXs (Condrea, 1974). Despite considerable research in this area, the specific mechanism(s) of action of the CTXs is(are) unknown (Chang, 1979; Harvey; 1985).

Phospholipase A₂ (PLA₂; EC3.1.1.4; ca. 12,000 MW) is a major component of bee and snake venom. PLA₂ is a Ca²⁺-dependent enzyme (Shipolini et al., 1971; Tsai et al., 1985) that catalyzes the hydrolysis of phospholipids at the #2 position releasing lysophospholipids and, primarily, unsaturated free fatty acids (Hanahan et al., 1960). The bee and cobra (*Naja naja*) venom PLA₂ enzymes readily hydrolyze biological phospholipid substrates, but are unable to penetrate membrane bilayers (Zwaal et al., 1975; Sundler et al., 1978; Fletcher et al., 1987). The inability to hydrolyze the inner phospholipids of the bilayer does not relate to interactions of these primarily negatively-charged phospholipids with spectrin (Raval and Allan, 1984), and can even be observed in pure phospholipid vesicles (Sundler et al., 1978; Wilschut et al., 1979). Hemolysis of fresh human red blood cells is not induced to any significant extent by bee venom PLA₂, even though almost all the phospholipids in the outer leaflet of the membrane bilayer have been hydrolyzed (Zwaal et al., 1975; Fletcher et al., 1987).

Louw and Visser (1978) reported that some CTX fractions were contaminated with trace amounts of venom PLA₂, which greatly potentiated the hemolytic activity of the CTX protein. Trace contamination of crude CTX preparations with PLA₂ activity has confounded the interpretation of some of the toxic actions of CTXs, especially when tested on hemolysis of erythrocytes (Harvey, 1985; Louw and Visser, 1978; Harvey et al., 1983). Highly purified CTXs are considered virtually devoid of PLA₂ contamination and have considerably reduced hemolytic potency compared to PLA₂ contaminated preparations. However, the potency of CTX on other preparations, such as skeletal muscle, is unaffected by PLA₂ contamination. Therefore, PLA₂ activity has been considered to be essential for the hemolytic action of CTXs on erythrocytes, suggesting the mechanism of action of CTX on the red blood cell does not reflect the same mechanism in skeletal muscle (Harvey, 1985). However, more recent studies have demonstrated that higher concentrations of PLA₂ do act in synergy with CTX in skeletal muscle and that the apparent difference in action of the CTXs may be related to differences in the concentration and type of free fatty acids in the two tissues in the absence of CTX (Fletcher and Lizzo, 1987), not differences in mode of CTX action.

One problem with determining the role of PLA₂ activity in the action of CTXs is that most investigators analyze PLA₂ contamination of CTX preparations by measuring the enzymatic activity on purified phospholipid or egg yolk substrates by various titration assays. Titration assays measure fatty acid release and cannot distinguish between PLA₁ (EC 3.1.1.32; removal of fatty acid from #1 position) and PLA₂ (removal of fatty acid from #2 position) activities. Additionally, titration assays are not very sensitive. CTX preparations that have no PLA activity on artificial substrates have been assumed to be enzymatically inactive on biological membrane systems such as the red blood cell. Rosenberg (1979) has cautioned against such assumptions

about PLA₂ activity and has suggested that PLA₂ activity must be directly determined on the biological substrate that is used for pharmacological or toxicological studies.

Snake venom CTX shares many of the properties of bee venom melittin, including interactions with PLA₂. The action of melittin on biological membranes has been better characterized than that of CTX, therefore melittin serves as a good model for CTX studies. Melittin, a low molecular weight (2,840) polypeptide of 26 amino acids, comprises about 50% of the dry weight of bee venom (Habermann, 1972). Among its toxic actions, melittin causes hemolysis of red blood cells and is cytolytic to other cell types (Habermann, 1972). Melittin enhances the rate of bee venom PLA₂ activity 5- to 6-fold on sonicated (Mollay and Kreil, 1974) and up to 300-fold on nonsonicated (Yunes et al., 1977) liposomes. Similar studies have not been conducted with CTX, despite its reported interaction with PLA₂. Understanding the mechanism by which melittin enhances bee venom PLA₂ activity is important, as melittin is used by investigators as a probe to activate tissue PLA₂ activity (Mollay et al., 1976; Shier, 1979). The use of melittin as a tissue PLA₂ activator evolved from the initial observations with bee venom and presumes some specificity to this action. Recently investigators have proposed that melittin enhances the rate of bee venom PLA₂ activity on multilamellar vesicles (nonsonicated liposomes) by converting these concentric bilayers into large unilamellar vesicles and thereby exposing a greater phospholipid surface to the enzyme (Dufourcq et al., 1986). However, these studies did not actually examine the interaction between melittin and PLA₂ and were conducted in the absence of Ca²⁺, which, in addition to supporting PLA₂ activity, normally binds to the headgroup of phospholipids and stabilizes membranes. The absence of this divalent cation could have contributed to the destabilizing effect of melittin on the multilamellar vesicles. Also, the conversion of multilamellar vesicles to large unilamellar vesicles does not explain the enhancement of bee venom PLA₂ on unilamellar substrates (sonicated liposomes) by melittin (Mollay and Kreil, 1974). However, melittin appears to cause local perturbations of bilayers (Dufourcq et al., 1986) that may somehow increase the access of PLA₂ to the phospholipid substrate. As with CTX, it is difficult to isolate melittin fractions that are free from contamination with venom PLA₂ activity (Mollay et al., 1976).

The relationship between the potency of CTX and the lipid composition of the target membranes is unclear (Condrea, 1979). It has been suggested that tissues with higher levels of free fatty acids are less sensitive to the interaction between CTX and PLA₂ (Fletcher and Lizzo, 1987). The relationship between free fatty acids and the response of membranes to CTX alone has not been directly examined.

The induction of contractures in skeletal muscle very possibly reflects an increase in free myoplasmic Ca²⁺ concentration. This increased myoplasmic free Ca²⁺ concentration would promote prolonged actin/myosin interactions. Indeed, leakage to one or more ions is suspected following CTX exposure (Chang, 1979). Cytoplasmic free Ca²⁺ concentrations can be monitored in lymphocytes with the use of fluorescent indicators, such as indo-1/AM and fura-2/AM (Gryniewicz et al., 1985).

Internalization is required for the action of a number of bacterial toxins (Middlebrook and Dorland, 1984). The rapid time to onset of effect (seconds; Fletcher and Lizzo, 1987) suggests signal transduction is a more likely mechanism for CTX action. Regardless the actual location of the toxin

molecule (inside or outside of the cell), it is important in developing prophylactic and therapeutic measures to determine accessibility of antibodies to the toxin site for neutralization.

Rationale:

Several model systems were used to examine: (1) the mode of action of snake venom CTXs; (2) the dependence of this action on the membrane lipid composition; (3) the interaction of CTX with PLA activity; (4) the effects of membrane composition on CTX action; (5) internalization of CTX, and; (6) the similarities and differences between CTXs and the presynaptically-acting snake venom PLA₂s. The advantages and disadvantages of each system are:

1. Skeletal Muscle. Contractures of skeletal muscle are believed to reflect the "true" cardiotoxic mechanism of CTXs. A special advantage of this preparation is that we have access to human skeletal muscle, which differs from the commonly used rat diaphragm preparation in Ca²⁺ dependence of CTX action (Fletcher and Lizzo, 1987). Disadvantages of skeletal muscle are the great degree of biological variability involved in examining contracture induction, the small number of studies that can be run in a single day and the inability to conveniently use fluorescence probes to directly monitor myoplasmic Ca²⁺ concentrations or patch-clamp electrophysiology to directly examine ion currents.
2. Erythrocytes. Despite some disagreement (Harvey, 1985), the action on red blood cells may reflect the "true" cardiotoxic mechanism of CTXs (Fletcher and Lizzo, 1987). The red blood cell has many advantages, including: it is a simple model system in which all the lipids are contained in the plasma membrane (no organelles in erythrocytes); red blood cells of different lipid composition can be obtained from a wide variety of species. The hemolysis assays with red blood cells allow many experiments to be conducted in a single day on the same batch of cells and they are far less subject to biological variability than skeletal muscle. The disadvantages of RBCs are the limited number of toxins that are active on them and their paucity of responses that can be examined, as apposed to excitable cells (muscle and nerve).
3. Lymphocytes. We have recently demonstrated lymphocytes to be a target for CTX action. Lymphocytes, like red blood cells, can be obtained from a large number of species. Patch-clamp electrophysiology for monitoring plasmalemmal ion currents and direct monitoring of cytoplasmic Ca²⁺ can be done with lymphocytes. Lymphocytes can be preloaded overnight with radiolabeled lipid precursors and subtle aspects of lipid metabolism examined the following day. Disadvantages of lymphocytes include their lack of availability (they must be isolated rapidly from fresh blood - free donors are scarce) and limited number of ion currents (primarily K⁺).
4. Platelets. Platelets are a rich source of tissue PLA₂ activity. Human platelets are readily available from the Red Cross.
5. Synaptosomes. Synaptosomes, or other nerve preparations, are essential for studies on presynaptically acting PLA₂ neurotoxins, as these toxins are specific for nerve tissue. Synaptosomes have the advantages that they provide a relatively large tissue mass for biochemical studies of presynaptic terminals impossible to obtain with phrenic nerve-diaphragm preparations, intrasynaptosomal Ca²⁺ can be monitored with fluorescent dyes and acetylcholine release can be monitored with radiolabeled

choline. The major disadvantage of synaptosomes is that they are not currently a good model for PLA₂ toxin studies. The reasons that they are not appropriate are: they have greatly elevated fatty acid levels that may mask subtle effect of PLA₂ toxins, and; the optimum conditions for simulating the action of the toxins at the neuromuscular junction have not been satisfactorily worked out. We are attempting to correct these problems.

6. Cell Lines. Cell lines provide homogeneous biological material. They can be rich in ion currents, depending on the cell type. The epithelial cells, like lymphocytes, have an advantage over muscle in that intracellular cytosolic Ca²⁺ concentrations can be monitored directly with fluorescence probes, such as indo-1. Additionally, patch-clamp electrophysiological studies can be conducted in which the effect of the toxin on specific membrane currents can be directly examined. Skeletal muscle patch-clamp studies require the use of cell culture, which might suppress expression of toxin sensitive channels (appears to be the case in cultured human and equine skeletal muscle; unpublished observations). The advantage in using both cell types is that epithelial cells have Ca²⁺ and voltage activated K⁺ channels and lymphocytes have K⁺ channels inhibited by Ca²⁺. Additionally, cell lines can be used for detailed analysis of lipid metabolism, as the phospholipid and neutral lipid pools can be readily radiolabeled overnight. Cell lines allow extensive lipid metabolism studies as they can be readily radiolabeled. The major disadvantages of cell lines are the time spent cloning and maintaining the cells and the "abnormal" responses that may be induced by either culture conditions or the process of immortalizing the cells.
7. Primary Cell Cultures. Primary cultures allow a far greater number of different patients and animals to be examined than cell lines.
8. Artificial Membranes. Artificial membranes can be prepared in many different forms, all of which yield different types of information regarding membrane perturbing agents. The membranes can be: mixed micelles, which are monolayers of phospholipid and a detergent (usually Triton X-100); unilamellar vesicles, which are bilayers of either a single phospholipid or mixture of phospholipids, and; multilamellar vesicles, which are concentric bilayers of phospholipids. These different membranes, all having different amounts of phospholipid exposed to the incubation medium, can be used to examine substrate availability and membrane penetration in PLA₂ studies. The primary disadvantage of these preparations is their dissimilarity in behavior to more complex biological membranes.

To examine if possible differences in mechanisms of action may exist in this group of toxins and the role of PLA₂ activity in hemolysis, we compared the effects of red blood cell age, incubation temperature and pH, divalent cation type and concentration and addition of glucose on hemolysis induced by three cardiotoxins from different snake venoms to that induced by bee venom PLA₂. Similarities in hemolytic behavior observed between the CTXs in the present study would suggest that the same or very similar mechanism within this group of toxins induces hemolysis. The dependence of this mechanism on contaminating PLA₂ activity can be examined by modifying the histidine residue at the catalytic site of contaminant PLA₂ with p-bromophenacyl bromide.

The effects of the toxin on lipid metabolism of the red blood cell,

primary cell cultures and cell lines can be directly examined using gas chromatographic analysis of free fatty acids. The effects of the toxin on endogenous PLA₂ activity of the red blood cell and lymphocyte can be directly examined using radiolabeled phospholipids. In this system the enzyme activity of red blood cells (or lymphocytes) alone, CTX alone and red blood cells (or lymphocytes) plus CTX can be determined. Highly detailed studies of effects on endogenous lipid metabolism can be conducted in primary cell cultures and cell lines with neutral and phospholipids preradiolabeled by feeding the cells ¹⁴C-fatty acids overnight.

The contract also examines the utility of purified phospholipid substrate systems for predicting the PLA activity of CTXs on biological membranes. In addition, the relationship between hemolysis of red blood cells promoted by two typical snake venom CTXs and one abnormally large CTX is compared to the hydrolysis of membrane bound phospholipids by these toxins to determine if PLA activity is crucial to the mechanism of red blood cell hemolysis. Since Sr²⁺ is known to be as effective as Ca²⁺ in supporting CTX contractures in skeletal muscle (Fletcher et al., 1981; Fletcher and Lizzo, 1987) and yet less effective than Ca²⁺ in supporting PLA₂ activity (Iwanaga and Suzuki, 1979), the effects of these two divalent cations on red blood cell hemolysis and phospholipid hydrolysis induced by the CTXs are contrasted.

To develop potential prophylactic and therapeutic agents, pharmacological studies examining antagonists of CTX action are conducted. These studies include the PLA₂ inhibitor mepacrine (quinacrine), the partial antagonists of CTX-induced skeletal muscle contractures dantrolene (Fletcher and Lizzo, 1987) and the divalent cation, Mn²⁺. Many of these studies were conducted in the first year and are included in the first Annual Report. A series of protein kinase C antagonists was also examined, as fatty acids (a product of lipolysis) activate protein kinase C (McPhail et al., 1984; Naor et al., 1988). Additionally pilot studies with a protein kinase C inhibitor, sphingosine, suggested that protein kinase inhibition might antagonize CTX action. Cross-inhibition of protein kinase C by calmodulin antagonists also resulted in these inhibitors being examined. These inhibitors were examined as regards hemolysis of red blood cells by CTX.

Since PLA₂ activity enhances the action of snake venom CTXs and bee venom melittin (Condrea, 1979), the effects of exogenously added fatty acids on CTX-induced hemolysis should be examined. These are highly toxic products of PLA₂ activity that account for the synergy observed between this enzyme and halothane, which has many of the properties of CTX, and PLA₂ (Fletcher et al., 1987). Additionally, the synergism between PLA₂ and the difficulty in preparation of either CTX or melittin fractions that are free of venom PLA₂, make it essential to find a means to test the toxicity of CTX and melittin under conditions that considerably reduce this contaminating PLA₂ activity. Treatment of the toxin fractions with p-bromophenacyl bromide inactivates, or greatly reduces, PLA₂ activity, without affecting the basic mechanism of the toxin (Fletcher et al., 1990d).

Assuming that hemolysis may be the end result of a series of events leading to leakiness of the cell, we sought to find a smaller molecule that might leak out long before hemoglobin. ³H-Deoxyglucose is taken up by cells and phosphorylated to deoxyglucose-6-phosphate. Deoxyglucose-6-phosphate is then trapped within the cell cytoplasm. Therefore, nonspecific cell leakage would result in the release of ³H-deoxyglucose-6-phosphate.

Since CTX is believed to increase ionic permeability (Chang, 1979), we

chose to examine the effects of CTX on lymphocyte and epithelial cell line cytoplasmic free Ca^{2+} . Additionally, in collaboration with Dr. Steven Wieland (Dept. Anatomy, Hahnemann University), we examined plasmalemmal ionic currents in both cell types using the whole cell variant of patch-clamp recording.

Internalization of the toxin can be determined in a number of ways. Our initial studies examined the patterns of phospholipid hydrolysis and free fatty acid release. Later studies examined the internalization of PLA_2 by the CTXs and melittin, as determined by the extent of hydrolysis of artificial bilayers. The synergistic interaction between CTX and PLA_2 can be examined using artificial substrates. Knowing that about 70% of the phospholipid is in the outer layer of liposomes, monitoring the extent of phospholipid hydrolysis can provide information on the ability of the PLA_2 to penetrate bilayers.

β -bungarotoxin (β -BTX) and other presynaptically-acting snake venom PLA_2 s exhibit a triphasic action on acetylcholine release from the phrenic nerve-diaphragm preparation (Chang, 1979). The observation of these phenomena is based on electrophysiological studies of events (EPPs, MEPPs) having durations of milliseconds. The relative insensitivity of even the most sensitive biochemical assays for acetylcholine requires that the transmitter be collected over periods of seconds to minutes. Therefore, extensive analysis of the time and concentration dependence of β -BTX action must be done in order to recreate in this biochemical model the same effects observed in the electrophysiological model. We have previously developed a crude method with which the second and third phases of the triphasic effect can be emulated in this biochemical model (Fletcher and Middlebrook, 1986). However, the model has to be refined, including application to a more highly purified synaptosomal fraction and improvement of the methods of isolation to reduce the extensive lipolysis that occurs during homogenization. Once this model has been sufficiently developed, then it can be applied to studies comparing the mechanisms of CTX action to those of the PLA_2 neurotoxins.

Experimental Methods:

Materials

Venom from *Naja naja atra*, CTX from *Naja naja kaouthia* venom (Lot# 125F-4007), bee venom PLA_2 (*Apis mellifera*), melittin, β -bungarotoxin, Tris base, Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Mes (4-morpholineethanesulfonic acid), bovine serum albumin (fatty acid free), 2'-7'-dichlorofluorescein, free fatty acid (methylated and unmethylated) standards, phospholipid, triglyceride and cholesterol standards, and butylated hydroxytoluene were purchased from Sigma Chemical Company (St. Louis, MO). CTX from *Naja naja atra* venom was purified from venom by ionic exchange chromatography (Jiang et al. 1989b). Venom from *Bungarus fasciatus* was a gift from Dr. T. Xue (Guangzhou Medical College, China). CTX from *Bungarus fasciatus* venom was purified, as previously described (Ji et al., 1983). The *N. n. kaouthia* and *N. n. atra* CTX fractions each appeared on SDS-PAGE slab gels as a single protein band of about 7,000 MW. The CTX from *B. fasciatus* venom appeared on the gels as a major band at approximately 16,000 MW and a minor band (<3% of the major band by scanning densitometry) at about 30,000 MW. The PLA_2 from *N. n. atra* venom was provided by Dr. Leonard Smith.

Hemolysis of Erythrocytes

Erythrocytes were obtained from the American Red Cross Blood Services and

stored as CPD whole blood (AS-1) at 4°C. These cells were used within about two weeks (plus or minus) of their expiration date. Hemolysis was determined, as previously described (Jiang et al., 1989a; 1989b; Fletcher et al., 1990b).

Treatment of Toxin Fractions with p-Bromophenacyl Bromide

Irreversible inactivation of PLA₂ activity was done as previously described (Jiang et al., 1989a).

Preparation of Mixed Micelle, Unilamellar and Multilamellar Substrates

Mixed micelles, unilamellar and multilamellar vesicles were prepared as described in the third Annual Report (1989-1990).

Gas Chromatographic Analysis of Fatty Acids and Phospholipids

Lipids were extracted from muscle (Fletcher et al., 1988; 1989; 1990c) or blood cells (Fletcher et al., 1987; Fletcher et al., 1990b) and the free fatty acids separated and analyzed by gas chromatography as their methyl esters, as previously described (Fletcher et al., 1987; 1988; 1989; 1990b; 1990c). Phospholipids were analyzed by the method of Bartlett (1959), as previously described (Fletcher et al., 1981; 1982; 1987; 1988; 1989; 1990b).

Determination of Phospholipase A₂ Activity

In some cases incubations were terminated by extracting and titrating the free fatty acids (Dole, 1956), as described in detail in the first Annual Report (1987-1988). When using radiolabeled substrates (see first Annual Report), the lipids of incubates were extracted (Fletcher et al., 1990a; 1990d) and neutral lipids were separated by 1-D TLC, as previously described (Fletcher et al., 1987). The plates were dried and the lanes scanned for radioactivity with a Raytest (Murray, PA) RITA Radio TLC Imaging Analyzer (Fletcher et al., 1990a; 1990d).

Radiolabeling Lipid Fractions in Skeletal Muscle Primary Cultures

Primary cultures of human and equine skeletal muscle were derived and maintained as previously described (Wieland et al., 1989). Cells are radiolabeled with ¹⁴C-linoleic acid for 3 days, as previously described for airway epithelial cell lines (Fletcher et al., 1990a; 1990d). The lipids are extracted, separated by 1-D TLC and radioactivity determined by scanning the plate, as previously described (Fletcher et al., 1990a; 1990d).

³H-Deoxyglucose Release from Erythrocytes Induced by CTX

³H-d-Glu-6-p release from erythrocytes was determined as previously described (Jiang et al., 1987; 1989b).

Lymphocyte and Epithelial Cell Line Isolation and Intracellular Ca²⁺ Determination (Indo-1/AM)

Whole venous blood was collected in heparinized tubes and lymphocytes processed within 2 hrs, as previously described (Gong et al., 1989; Fletcher et al., 1990e). Lymphocytes and epithelial cell lines were loaded with indo-1/AM using standard methodology (Grynkiewicz et al., 1985), as previously described (Gong et al., 1989; Fletcher et al., 1990a; 1990e).

Membrane Currents Using the Whole Cell Variant of Patch Clamp Electrophysiology

Cells were voltage-clamped at room temperature (21-23°C) using the whole-cell variant of the patch clamp method previously described (Wieland et al., 1987; 1989; Gong et al., 1989).

Preparation of Synaptosomes and Determination of Acetylcholine (ACh Release) and Choline Uptake

Studies were done using the crude P₂ pellet preparation, as previously described (Fletcher and Middlebrook, 1986), or the synaptosomal preparation described by Dunkley et al. (1988). These methods are described in detail in the second and third Annual Reports (1988-1989; 1989-1990). Choline uptake was performed basically as previously described (Fletcher and Middlebrook, 1986), except that the synaptosomes were filtered, as described in the third Annual Report (1989-1990).

Results:

PROBLEM 1. What are the effects of CTX alone and CTX-PLA₂ combinations on contracture induction of human and rat skeletal muscle and on Ca²⁺ transients in human and equine lymphocytes?

The complete dose-response relationships of *Naja naja kaouthia* CTX in human and rat skeletal muscle and the enhancement of CTX-induced contractures by PLA₂ were previously reported (Fletcher and Lizzo, 1987). The *Naja naja atra* CTX appears about half the potency of the *Naja naja kaouthia* CTX (see first Annual Report). Quinacrine (mepacrine), a tissue phospholipase A₂ antagonist, had no effect on contractures in skeletal muscle induced by CTX (*N. n. kaouthia*) (first Annual Report). Quinacrine does not antagonize (and may slightly increase in the case of humans) the action of CTX. Therefore, although free fatty acids potentiate the action of CTX, there is an action of the toxin on skeletal muscle completely independent of endogenous free fatty acid production. However, this conclusion is based on the supposition that quinacrine is a specific PLA₂ inhibitor, and this is not likely (Fletcher et al., 1987).

The *B. fasciatus* CTX (0.5-2 µM) enhances Ca²⁺-activated K⁺ channels in human epithelial cell lines and inhibits Ca²⁺-inhibited K⁺ channels in lymphocytes (Gong et al., 1989). In both cell types intracellular Ca²⁺ level perturbations by the CTX appeared shorter-lived than the effects on K⁺-channel function (Gong et al., 1989). The *N. n. kaouthia* CTX did not cause Ca²⁺ transients (Gong et al., 1989). Unlike the transient and reproducible increase in cytoplasmic Ca²⁺, the effect on the K⁺ channels is irreversible. The toxin appears to constantly activate a Ca²⁺-dependent process.

PROBLEM 2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?

The complete dose-response for the *N. n. kaouthia* CTX has been compared in erythrocytes with different phospholipid distributions and levels of cholesterol (Fletcher et al., 1990b). The phospholipid distributions as determined by us in these species are similar to those reported by Nelson (1967). Also, the cells differ in free fatty acids and in fatty acids released from phospholipids by venom PLA₂s (Fletcher et al., 1990b). The order of species susceptibility to hemolysis by CTX is related to the amount of

saturated free fatty acid prior to PLA₂ treatment. In contrast, there is no relationship between the levels or patterns of free fatty acids after PLA₂ treatment and the susceptibility to hemolysis by the CTXs.

The *N. n. kaouthia* and *N. n. atra* CTXs are potentiated by exogenously added unsaturated fatty acids (oleic and arachidonic acid) in Ca²⁺ and Sr²⁺ media (Fletcher et al., 1990b). The *B. fasciatus* CTX is much less affected by the unsaturated fatty acids. An 18 carbon saturated fatty acid, stearic acid (1-40 μM; identical to oleic, but no double bond) does not increase hemolysis by any of CTXs (Fletcher et al., 1990b).

On mixed micelle substrates PLA₂ activity from bee venom exhibited a substrate specificity that favored polyunsaturated fatty acids at the #2 position (Fletcher et al., 1990d). The activity on PC with saturated fatty acids at position 1 and 2 (dipalmitoyl PC) was lowest. Egg yolk PC, which is a mixed molecular species of substrate primarily comprised of 16:0, 18:1 (R₁, R₂) and 16:0, 18:2 (Porter et al., 1979), was next in preference and a pure unsaturated (#2 position) substrate was the most preferred.

PROBLEM 3. What are the hemolytic effects of CTX alone and CTX-PLA₂ combinations in red blood cells from different species?

Freshly collected human red blood cells are relatively resistant to hemolysis induced by CTXs (Condrea, 1974; 1979). However, aged human red blood cells, in which ATP levels have been depleted, are suitable substrates for hemolysis by a basic PLA₂ from *Naja nigricollis* snake venom (Condrea et al., 1980) with cardiotoxin-like properties (Lee et al., 1977; Fletcher et al., 1982). The potency of *N. n. kaouthia* CTX was greatly enhanced after 4 days of incubation of the red blood cells in HEPES buffer (Jiang et al., 1989a). Therefore, aged human red blood cells were used in many of the studies to optimize levels of hemolysis. Hemolysis induced by the CTXs was clearly temperature dependent. The potencies of all three toxins were greater at 37°C than at 20°C (Jiang et al., 1989a). The *N. n. kaouthia* CTX showed a greater degree of hemolysis in Ca²⁺ medium relative to Sr²⁺ or Ba²⁺ medium (Jiang et al., 1989a; 1989b) beyond 3 μM CTX concentration. At a 2 mM concentration, Sr²⁺ was about equipotent to Ba²⁺ in promoting hemolysis by the three CTXs at all toxin concentrations (Jiang et al., 1989a). At low concentrations (< 1 mM) Ca²⁺ enhanced hemolysis due to all three CTXs relative to cation-free media (Jiang et al., 1989a). Beyond about 2.5 mM all the divalent cations exerted increasingly inhibitory effects compared to hemolysis by the three CTXs in a divalent cation-free medium (Jiang et al., 1989a). The *B. fasciatus* CTX was unique among the CTXs in that high concentrations of Ca²⁺ (> 40 mM) were actually stimulatory in regard to hemolysis (Jiang et al., 1989a). The three CTXs were greatly affected by the pH of the medium, exhibiting a sharp increase in hemolytic activity between pH 7.5 and 8.5 in a Ca²⁺ containing medium (Jiang et al., 1989a). Glucose has been demonstrated to reduce hemolytic activity of certain venom PLA₂s in a medium containing high levels of Ca²⁺, possibly by maintaining levels of ATP (Martin et al., 1975). In a medium with a low (2 mM) concentration of Ca²⁺ or Sr²⁺, glucose increased the activity of the three CTXs (Jiang et al., 1989a). Similar effects of glucose were observed in a Ba²⁺ (2 mM) containing medium for the *N. n. kaouthia* and *N. n. atra* CTXs. However, this concentration of Ba²⁺ inhibited hemolysis by the *B. fasciatus* CTX (Jiang et al., 1989a). The effect of glucose at a cation concentration of 40 mM was to slightly reduce hemolytic activity of all three

CTXs (Jiang et al., 1989a). Treatment with *p*-bromophenacyl bromide for 20 hrs completely abolished PLA₂ activity of bee venom PLA₂ and *B. fasciatus* CTX (first Annual Report). However, this treatment had very little effect on hemolysis induced by *N. n. kaouthia* or *B. fasciatus* CTXs (Jiang et al., 1989a; first Annual Report).

The order of potency was the same for ³H-dGlu-6-p release from human red blood cells (Jiang et al., 1989b). Five different divalent cation conditions (none, Ca²⁺, Sr²⁺, Ba²⁺, Mn²⁺) had similar effects on hemolysis and ³H-dGlu-6-p release at two concentrations of *N. n. kaouthia* CTX (Jiang et al., 1989b). The effects of varying the concentration of Ca²⁺ on ³H-dGlu-6-p release and hemolysis were also similar (Jiang et al., 1989a; 1989b). As with hemolysis (Jiang et al., 1989a), ³H-dGlu-6-p release induced by *N. n. kaouthia* CTX (3 μM) exhibits a sharp increase in the range of pH 7.0 to 8.5 (Jiang et al., 1989b). There were no dramatic differences in the time courses of release of these two markers of CTX action (Jiang et al., 1989b).

In order to further examine the possible role of trace contamination of the toxin fraction with venom PLA₂ in the hemolytic action of the *N. n. kaouthia* CTX, we treated the toxin fraction with *p*-bromophenacyl bromide (*p*-BPB), as previously described (Jiang et al., 1989a). This treatment with *p*-BPB abolished the stimulatory effect of Ca²⁺ at higher concentrations of *N. n. kaouthia* CTX, making the toxin dose-hemolysis curves (Jiang et al., 1989b) identical whether done in Ca²⁺-containing or Ca²⁺-free media.

Bee venom PLA₂ and snake venom PLA₂ potentiate the hemolytic action of the *N. n. kaouthia* and *N. n. atra* CTXs (first Annual Report). The hemolytic activity of the *B. fasciatus* CTX was not greatly affected by the presence of bee venom PLA₂. Replacing Ca²⁺ with Sr²⁺ did not affect the degree of hemolysis by the combination of bee venom PLA₂ and *N. n. kaouthia* CTX or bee venom PLA₂ and *N. n. atra* CTX (first Annual Report). However, Sr²⁺ did reduce the hemolytic activity of the *N. n. kaouthia* CTX and *N. n. atra* CTX when added in the absence of bee venom PLA₂. In contrast to the results obtained with bee venom PLA₂, the potentiation between snake venom PLA₂ and *N. n. kaouthia* or *N. n. atra* CTXs was antagonized by Sr²⁺. Agkistrotoxin (AGTX) is a single chain specific presynaptic neurotoxin with PLA₂ activity, but with no hemolytic activity (unpublished observations). Addition of either AGTX or *B. fasciatus* CTX along with *N. n. kaouthia* CTX results in greatly increased levels of hemolysis. The interaction of *N. n. kaouthia* CTX with AGTX is almost completely abolished in a Sr²⁺ medium, whereas that with *B. fasciatus* CTX is only partially antagonized under the same condition (first Annual Report).

We examined the kinetics of hemolysis by the snake venom CTXs. The increased susceptibility of aged red blood cells to lysis by the CTXs is the result of an increase in V_{max} for the first 4 days, but a decrease in K_m in addition to the increase in V_{max} after 8 days (second Annual Report). The effects of pH can be attributed to both an increase in V_{max} and K_m (second Annual Report). In the presence of Ca²⁺ there appears to be a break in the dose-response curve above about a 3 μM concentration of CTX. This break can be attributed to an increase in both K_m and V_{max}. In a Ca²⁺ medium the K_m is about 5 μM at low concentrations of CTX. This is almost 10-fold higher than observed in a Ca²⁺-free medium (0.45 μM), in agreement with Ca²⁺ competing with CTX for binding (second Annual Report). The K_m values for Sr²⁺ and Ba²⁺ are about 1 μM and 1.25 μM, respectively, suggesting that these ions may compete with CTX for binding, but are not as effective antagonists as Ca²⁺.

A series of inhibitors of protein kinase C and calmodulin were examined

as regards the hemolytic action of CTXs. Sphingosine and polymyxin B both inhibited hemolysis by the *N. n. kaouthia* CTX at low μM concentrations of antagonist (second Annual Report). Trifluoperazine, chlorpromazine, phloretin, and staurosporin were also ineffective as antagonists of CTX action.

The p-BPB-treated *N. n. atra* CTX did not have the Sr^{2+} -supported hemolytic activity observed with the *N. n. kaouthia* CTX (third Annual Report). In contrast, the p-BPB-treated *N. n. kaouthia* CTX had a Sr^{2+} -supported hemolytic activity evident even after p-BPB treatment. This implies that the *N. n. atra* CTX has a much greater dependence on venom PLA_2 contamination for its action than does the *N. n. kaouthia* CTX (third Annual Report).

PROBLEM 4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?

In addition to the studies on CTX, we have been conducting studies on another cardiotoxin-like peptide (melittin) from bee venom. Melittin has been reported to activate bee venom (Mollay and Kreil, 1974) or endogenous (Mollay et al., 1976) PLA_2 activity. Some inconsistencies have been observed with melittin as an activator of endogenous PLA_2 (Metz, 1986; Rosenthal and Jones, 1988).

Effects of melittin on endogenous PLA_2 activity in airway epithelial cells

Three different cell lines were radiolabeled with ^{14}C -fatty acid prior to beginning the experiments (Fletcher et al., 1990d). Bee venom PLA_2 caused a significant decrease in total phospholipid corresponding to the increased free fatty acid (Fletcher et al., 1990d). The native melittin fraction caused greater hydrolysis of phospholipids than did bee venom PLA_2 . The percentage of bee venom PLA_2 contamination in the melittin fraction was estimated to be about 0.1% based on previous studies. Pretreatment of the melittin fraction with p-BPB decreased free fatty acid production by 75% (Fletcher et al., 1990d). When bee venom PLA_2 is added to the p-BPB-treated fraction at an amount approximately equal to the estimated contamination in the native melittin fraction, phospholipid hydrolysis returns to the levels of the native melittin fraction. Therefore, at least 75% of the " PLA_2 activation" by melittin can be attributed to contamination of the fraction with bee venom PLA_2 .

Effects of CTX on endogenous PLA_2 and other lipolytic activities in primary cultures of skeletal muscle

Human primary muscle cell cultures were radiolabeled with ^{14}C -fatty acid prior to beginning the experiments (third Annual Report). We found that the most consistent results in cell cultures of skeletal muscle are obtained with a concentration of 10 μM *Naja naja kaouthia* CTX and a 2 hr incubation period (third Annual Report). Both the native and p-bromophenacyl bromide (p-BPB)-treated *Naja naja kaouthia* snake venom CTXs cause the production of diacylglyceride and free fatty acid in primary cultures of skeletal muscle from normal or malignant hyperthermia susceptible patients to about the same extent. Thus, the snake venom PLA_2 contamination in the native CTX fraction is insignificant in this system. Normal patients exhibit a decrease in phospholipid labeling; whereas the malignant hyperthermia patients exhibit a decrease in triglyceride labeling (third Annual Report). The effects on triglyceride metabolism appear to relate specifically to the malignant

hyperthermia disorder. The importance of reducing the venom PLA₂ contamination in the CTX fraction can readily be seen with the less highly purified CTX from *Naja naja atra* snake venom (third Annual Report). The p-BPB treatment of the fraction dramatically reduced PLA₂ activity. In general, normal equine muscle cells are similar to normal human cells in response to the CTX fractions (third Annual Report). In contrast, cells from a horse with hyperkalemic periodic paralysis did not exhibit the production of diglyceride, but did exhibit the increase in free fatty acids (third Annual Report). While the results are rather preliminary, it appears that melittin has a slightly different effect on normal muscle than does snake venom CTX (third Annual Report). First, the triglyceride decrease seen only in malignant hyperthermia muscle with CTX is also seen in normal muscle with melittin. Next the ratio of free fatty acid production to diglyceride production by melittin is greater in muscle from malignant hyperthermia patients than normals.

Effects of CTX on hydrolysis of red blood cell phospholipids

We examined the hydrolysis of red blood cell phospholipids by fractions of bee venom PLA₂ (BVPLA₂), snake venom (*N. n. atra*) PLA₂ (SVPLA₂), *N. n. kaouthia* CTX (NnkCTX), *N. n. atra* CTX (NnaCTX) and p-BPB-treated derivatives of the CTXs (NnkCTXB, NnaCTXB). The PLA₂s and untreated CTX fractions (especially that from *N. n. atra* venom) caused extensive hydrolysis of phospholipids, as evidenced by very high levels of fatty acids relative to preparations not exposed to toxin (third Annual Report). Inactivation of the PLA₂ contamination in the CTX fractions by treatment with p-BPB totally abolishes any PLA₂ activity on red blood cells, suggesting either: (1) CTX does not activate endogenous PLA₂ enzymes, or (2) there is no PLA₂ enzyme in red blood cells for the toxin to activate.

PROBLEM 5. What is the role of toxin internalization in the action of CTX?

To address internalization in the action of CTXs, we examined penetration of bilayers by PLA₂ in the absence or presence of either melittin or CTX. Penetration was assessed by greater than 70% hydrolysis of the unilamellar vesicle substrate, as 70% of the phospholipid is in the outer layer of these vesicles. We examined the effects of melittin on an arachidonic acid containing substrate embedded in an egg yolk PC:Triton X-100 matrix, or in MLVs or ULVs. Radiolabeled L-3-PC (1-stearoyl-2-[1-¹⁴C]arachidonyl) was used to determine the level of hydrolysis in all cases.

Effects of melittin on bee venom PLA₂ activity

All of the studies were done with p-BPB-treated melittin. We first examined the effects of PLA₂ concentration on the Ca²⁺ and NaCl-dependent melittin stimulation of the hydrolysis of mixed micelles. There was no stimulation of PLA₂ activity by melittin in the presence of Ca²⁺ alone (i.e., in the absence of NaCl), despite a range of phospholipid hydrolysis values in the absence of melittin of about 5 to 45% (third Annual Report). In contrast, melittin did stimulate bee venom PLA₂ activity on mixed micelles under the following conditions (third Annual Report): 1) a NaCl concentration of 130 mM; 2) a PLA₂ concentration > 5 nM (phospholipid hydrolysis > 15%) and; 3) a concentration of melittin of 10 μM.

We observed an increase in amount of hydrolysis on ULVs and MLVs with increasing concentrations of PLA₂ and attributed this to one of four

possibilities: 1) PLA₂ enzymes can penetrate bilayers, despite a large body of literature indicating otherwise; 2) incomplete hydrolysis had occurred (not the maximum extent) due to too short an incubation time; 3) the PLA₂ fractions may contain trace contamination with melittin; and 4) nonproductive binding occurs that inactivates the enzyme. We examined the consequence of an increased incubation time on bee venom PLA₂ activity on a ULV substrate. The activity appeared to have already reached a plateau at 2 hr for a fixed concentration of PLA₂, suggesting that the PLA₂ enzyme does not penetrate the membrane bilayer and that we were not dealing with incomplete hydrolysis (third Annual Report). However, the amount of hydrolysis increased with increasing enzyme concentration, defying rules of normal enzyme kinetics. We conclude that trace melittin contamination in the bee venom PLA₂ fraction would be insufficient to allow the enzyme to penetrate the bilayer, based on the high concentration of melittin relative to PLA₂ required for increased hydrolysis (third Annual Report). Therefore, nonproductive binding most likely accounts for this phenomenon. The results obtained with ULVs and radiolabeled substrates consistently demonstrate the enhancement of PLA₂ activity by melittin under every condition examined. Ca²⁺ was not essential for this action of melittin (third Annual Report). In examining the time course of PLA₂ activity on ULVs, we observed that the most consistent results were obtained when the specimens were immediately extracted completely (third Annual Report).

Effects of snake venom CTX on bee venom and snake venom PLA₂ activities and of melittin on snake venom PLA₂ activity

We tested whether CTX shared the synergism with PLA₂ observed with melittin (third Annual Report). At low concentrations of bee venom PLA₂ (≤ 10 nM), CTX greatly increased the enzymatic activity of the bee venom PLA₂. However, at higher concentrations of PLA₂ (≥ 100 nM), CTX had no effect on phospholipid hydrolysis. There was no apparent CTX-facilitated penetration of the membrane bilayer at high concentrations of the bee venom enzyme, in agreement with melittin.

We compared the maximum extent of phospholipid hydrolysis by *Naja naja atra* PLA₂ to that of bee venom PLA₂ and found that the concentration of the snake venom enzyme had to be about 10-fold greater than the bee venom enzyme for hydrolysis of the entire outer leaflet of the membrane bilayer (third Annual Report). CTX had very little effect on high concentrations of snake venom PLA₂, in agreement with bee venom PLA₂ (third Annual Report). The maximum extent of hydrolysis was less for the snake venom PLA₂ in the presence of CTX, than in the presence of melittin. Examining the effects of NaCl and Ca²⁺ on the interaction between high concentrations of snake venom PLA₂ (1 μ M) and CTX revealed that these cations increase the maximum extent of hydrolysis. In the presence of Ca²⁺ and/or NaCl, CTX did not further enhance the hydrolysis of ULVs by the snake venom enzyme. We conclude that at high concentrations of PLA₂, CTX only seems to replace cations.

The PLA₂ from *Naja naja atra* snake venom exhibits the same concentration-dependent degree of hydrolysis we previously observed for bee venom PLA₂ (third Annual Report). At a concentration of 3 μ M, bee venom PLA₂ is capable of hydrolyzing the ULVs to a greater extent than snake venom PLA₂. Unlike bee venom PLA₂, which was unaffected by BSA, snake venom PLA₂ activity on ULVs is greatly enhanced by the presence of 0.5% BSA in the incubation medium. The interaction of melittin with bee venom PLA₂ was highly dependent on NaCl in

the medium and independent of Ca^{2+} . The opposite is true for snake venom PLA₂ and CTX. In the absence of BSA, NaCl antagonized the enhancement of snake venom PLA₂ activity by *Naja naja kaouthia* CTX. The presence of Ca^{2+} overcame the inhibitory effect of NaCl.

PROBLEM 6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA₂s?

The major accomplishment with regard to this problem was the development of methodologies to facilitate addressing this problem in a future grant. Most of this work was reported in the third Annual Report. We were not completely satisfied with using purified synaptosomal preparations for acetylcholine release assays. There was considerable variation in yield and in viability from batch to batch. The most exciting finding was that choline uptake may be as good or a better model of toxin action than acetylcholine release. *Effects of neostigmine and nonradiolabeled choline on ^{14}C -choline and ^{14}C -acetylcholine release*

Discussion and Conclusions:

PROBLEM 1. What are the effects of CTX alone and CTX-PLA₂ combinations on contracture induction of human and rat skeletal muscle and on Ca^{2+} transients in human and equine lymphocytes?

The cobra venom CTXs cause relatively rapid (ca. 1 min.) contractures in skeletal muscle (Fletcher and Lizzo, 1987). This is in contrast to the much longer time to hemolysis (30 and 120 min for 10 and 20% hemolysis, respectively; Jiang et al., 1989b). Other long-term effects of the cobra venom CTXs include decreasing the indirectly evoked muscle twitch of skeletal muscle in the presence of curare (Fletcher et al., 1981) and increasing diglyceride and free fatty acid levels in skeletal muscle cell cultures (third Annual Report). Therefore, it appears that cobra venom CTXs have at least two different mechanisms of action. The first is an almost immediate action, as manifest by contractures of skeletal muscle (Harvey, 1985; Fletcher and Lizzo, 1987). The second may be the result of increases in free fatty acids and diglycerides and takes a much longer time to occur. The rapid action is transient and the preparation completely recovers. The second action has a prolonged latency and causes cell death. Since there are two mechanisms involved, this may explain why Harvey (1985) correctly concluded that CTX action on skeletal muscle contractures differs from that on red blood cells. However, it is unclear as to which mechanism is most important in causing death. Most likely the first mechanism is most important at high concentrations of toxins and the second mechanism is more important at low concentrations. Since the first mechanism is transient, extremely high concentrations of toxin would be required for a very rapid death (<1-5 min). Therefore, most probably the second (delayed) mechanism of toxin action is of the greatest concern with regard to toxicity, as death is not that rapid with these toxins. Therefore, in contrast to the conclusions of Harvey (1985), the best models of toxin action would be hemolysis, or the production of free fatty acids and diglycerides in skeletal muscle cell cultures. Skeletal muscle contractures should still be studied, however, as they represent a second potential cause of death.

A PLA₂ from *B. fasciatus* snake venom with CTX-like properties also causes contractures and slight hemolysis of red blood cells (Gong et al., 1989). This toxin irreversibly regulates Ca²⁺-modulated K⁺ currents, but not by maintaining high levels of Ca²⁺ in the cell (Gong et al., 1989).

PROBLEM 2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?

Since the dose-response for *N. n. kaouthia* CTX was the same for human and rat skeletal muscle (Fletcher and Lizzo, 1987), we did not pursue extensive lipid analyses of these tissues to determine the effects of lipid composition on CTX action. However, the hemolytic potency of CTXs did vary considerably for red blood cells from different species (Fletcher et al., 1990b). These erythrocytes differed considerably in many of the lipid components (Fletcher et al., 1990d). While there was some relationship between the amount of phosphatidylcholine in the membrane and the susceptibility to hemolysis, the one consistent finding in the five species examined was an inverse relationship between the amount of free saturated fatty acid in the membrane and the extent of hemolysis. That is, the more saturated fatty acid in the membrane, the less susceptible the membrane was to hemolysis. As regards interactions with venom PLA₂s, CTX-induced hemolysis is potentiated by exogenously added unsaturated fatty acids (products of PLA₂ activity) (Fletcher et al., 1990b). Even on purified substrates such as mixed micelle, the activities of toxins, in this case bee venom PLA₂ phospholipid hydrolysis, are dependent on fatty acid (actually fatty ester) composition.

PROBLEM 3. What are the hemolytic effects of CTX alone and CTX-PLA₂ combinations in red blood cells from different species?

The factors affecting hemolysis by the cobra CTXs are erythrocyte age, temperature, divalent cations, pH and the presence of glucose. The release of the relatively small (relative to hemoglobin) molecule deoxyglucose-6-phosphate paralleled hemoglobin release under all conditions, suggesting that a subpopulation of older cells are selectively lysed, as opposed to partial lysis of all cells.

The *N. n. kaouthia* CTX has a very distinct hemolytic component that is totally independent of PLA₂ activity. In contrast the CTX from *N. n. atra* venom is highly dependent on PLA₂ activity (venom contamination) to exert any hemolytic activity. Therefore, the mechanism of action of some CTXs might be attributed as solely due to the PLA₂ contamination in the fraction, but this is not true of all CTXs. An important point is that CTXs allow penetration of otherwise nonpenetrating PLA₂s into the membrane bilayer (see below). PLA₂ activity is not toxic unless it penetrates into the inner leaflet of the membrane bilayer.

Some, but not all, protein kinase C inhibitors antagonize the hemolytic activity of the cobra CTXs. We are not certain what this means. It may suggest that the phosphatidylserine binding site of these antagonists is important for toxin action. Alternatively, the two effective protein kinase C antagonists (sphingosine and polymyxin B) act by a mechanism independent of protein kinase C inhibition.

PROBLEM 4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?

CTX and bee venom melittin have both been suggested to activate endogenous PLA₂ activity. Bee venom melittin fractions from commercial sources contain enough bee venom PLA₂ contamination to account for about 75% of what was formerly presumed to be activation of endogenous (tissue) PLA₂ activity (Fletcher et al., 1990d). The studies of melittin and bee venom PLA₂ interactions on purified substrates demonstrate that the ideal potentiating capability of CTX and melittin is at about the percentage of PLA₂ that is normally found as contamination. The remaining fatty acid production (about 25% of the total induced by melittin) also does not appear to be derived from tissue PLA₂ activity, but either from activation of *de novo* synthesis of free fatty acids, or from triglyceride breakdown (triglyceride lipase activation) (third Annual Report). These latter studies were conducted with synthetic melittin to rule out the contribution of any venom PLA₂.

Both the native and p-bromophenacyl bromide (p-BPB)-treated *Naja naja kaouthia* snake venom CTXs cause the production of diacylglyceride and free fatty acid in primary cultures of skeletal muscle from normal or malignant hyperthermia susceptible patients to about the same extent. Thus, the trace snake venom PLA₂ contamination in the *N. n. kaouthia* CTX fraction does not cause the elevated free fatty acids. In contrast, the greater contamination with venom PLA₂ in the *N. n. atra* fraction greatly contributes to fatty acid production. The CTXs have effects that can vary in the presence of muscle disorders linked to fatty acid metabolism (malignant hyperthermia and hyperkalemic periodic paralysis).

Also supporting an action of *N. n. kaouthia* CTX completely independent of PLA₂ activity was the total lack of hydrolysis in red blood cells under conditions in which the toxin caused considerable hemolysis. Therefore, even in red blood cells, the *N. n. kaouthia* CTX does not require PLA₂ activity for its action.

PROBLEM 5. What is the role of toxin internalization in the action of CTX?

The long latency until toxin action suggests that, at least for the mechanism of toxin action with a longer latency to onset (see PROBLEM 1), internalization of toxin may be very important. To address internalization in the action of CTXs, we examined penetration of artificial phospholipid bilayers (unilamellar vesicles; ULVs) by PLA₂ in the absence or presence of either melittin or CTX. Melittin and CTX both allowed very low concentrations of either bee venom PLA₂ or snake venom PLA₂ to penetrate to the inner leaflet of membrane bilayers. The interaction between melittin and bee venom PLA₂, requires the presence of NaCl, but not Ca²⁺. The opposite is true for snake venom PLA₂ and CTX, as NaCl antagonized the enhancement of snake venom PLA₂ activity by *Naja naja kaouthia* CTX. The presence of Ca²⁺ overcame the inhibitory effect of NaCl. Melittin stimulated bee venom PLA₂ activity on mixed micelles, in which the substrate is 100% available to the enzyme, under the following conditions: 1) a NaCl concentration of 130 mM; 2) a PLA₂ concentration > 5 nM (phospholipid hydrolysis > 15%) and; 3) a concentration of melittin of 10 µM. Nonproductive binding most likely occurs at low concentrations of PLA₂. Unlike bee venom PLA₂, which was unaffected by BSA, snake venom PLA₂ activity on ULVs is greatly enhanced by the presence of BSA

in the incubation medium. Therefore, while bee venom melittin and snake venom CTXs share similar properties, there are considerable differences in their exact mechanisms of action.

PROBLEM 6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA₂s?

The major accomplishment with regard to this problem was the development of methodologies to facilitate addressing this problem in a future grant. Choline uptake may be as good or a better model of toxin action than acetylcholine release.

Conclusions

Our studies suggest that CTXs have at least two mechanisms of action. The first has a rapid onset and is reversible. The second has a prolonged latency to onset and results in cell death (cytolytic action). Free fatty acids in the membrane markedly affect CTX action, with saturated fatty acids making a membrane much more resistant to the CTX action. Melittin and CTX act by similar, but not identical mechanisms, based on similarities and differences in their interactions with PLA₂ and differences in effects on lipid metabolism in cell cultures. Certainly bee venom and snake venom PLA₂s differ markedly in their interactions with CTXs and melittin. The most important and consistent interaction (marked mutual synergism) between the membrane perturbing toxins (CTX and melittin) and any PLA₂ is allowing the PLA₂ enzyme to penetrate to formerly unavailable substrates. The *Naja naja kaouthia* CTX and melittin *do* possess activities *not* dependent on venom PLA₂ contamination in the toxin fraction. These toxins both elevate free fatty acids and diglycerides by a mechanism unrelated to activation of endogenous PLA₂ activity. At this time we believe that these elevated neutral lipids are related to activation of *de novo* synthesis of fatty acids, or breakdown of triglycerides, by the CTXs.

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Jiang, M.-S., Fletcher, J.E. and Smith, L.A. (1989) Factors influencing the hemolysis of human erythrocytes by cardiotoxins from *Naja naja kaouthia* and *Naja naja atra* venoms and a phospholipase A2 with cardiotoxin-like activities from *Bungarus fasciatus* venom. *Toxicon* 27, 247-257.

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MANUSCRIPTS IN PREPARATION

1. SPHINGOSINE AND POLYMYXIN B ARE COMPETITIVE INHIBITORS OF HEMOLYSIS OF HUMAN ERYTHROCYTES BY NAJA NAJA KAOUTHIA SNAKE VENOM CARDIOTOXIN, coauthored by Jeffrey E. Fletcher and Ming-Shi Jiang.
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